Photobacterium damselae subsp. damselae, an emerging fish pathogen in the Black Sea: evidences of a multiclonal origin

Mateus S. Terceti¹, Hamdi Ogut² & Carlos R. Osorio¹*

¹Instituto de Acuicultura, Universidade de Santiago de Compostela, Santiago de Compostela, Galicia, Spain;
² Bursa Technical University, Faculty of Natural Sciences, Architecture and Engineering, Department of Bioengineering, Bursa, Turkey

Running Title: P. damselae subsp. damselae in the Black Sea

*For correspondence:
Carlos R. Osorio
Departamento de Microbiologia e Parasitologia, Instituto de Acuicultura, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain
Email: cr.osorio@usc.es
Tel. (+34) 881 816050
Fax. (+34) 881 816047

Copyright © 2016, American Society for Microbiology. All Rights Reserved.
ABSTRACT

Photobacterium damselae subsp. damselae is considered an emerging pathogen of marine fish of importance in aquaculture, with a notable increase in its geographical distribution during the last years. In this study we carry out for the first time a genetic and pathobiological characterization of 14 strains isolated from sea bass (Dicentrarchus labrax) reared in the Southeastern Black Sea, where high mortalities were observed at two aquaculture farms during the summer and autumn of 2011. Heterogeneity was evidenced among strains in phenotypical traits as sucrose fermentation, motility and hemolysis. Although 11 out of 14 isolates were hemolytic, we found that all the isolates lacked the pHDD1 virulence plasmid that encodes the phospholipase-D damselfish (Dly) and the pore-forming toxin PhlyP, two hemolysins previously reported to constitute major virulence factors for turbot. Subsequent PCR and sequencing analyses demonstrated that the 11 hemolytic isolates harboured a complete hlyA<sub>ch</sub> gene, a chromosome I-borne gene that encodes HlyA<sub>ch</sub> hemolysin, whereas the three non-hemolytic isolates contained hlyA<sub>ch</sub> pseudogenes caused by insertion sequence elements. Virulence challenges with two representative strains revealed that, albeit less virulent than the pHDD1-harbouring strain RM-71, both the plasmidless hlyA<sub>ch</sub>-positive and hlyA<sub>ch</sub>-negative Black Sea isolates were pathogenic for sea bass. A phylogenetic analysis based on the toxR gene sequence uncovered a greater diversity in the isolates, indicating that presence of this pathogen in the Black Sea was not caused by the introduction and spread of a single virulent clone but by the proliferation of different clones.

IMPORTANCE

The geographical distribution of marine bacterial pathogens is undergoing a worldwide increase. In particular, bacteria of the group of Vibrios are increasingly being isolated as causative agents of disease in novel species of cultivated fish, in areas where they had not been previously reported. Here we characterize for the first time a collection of isolates of the fish and human pathogen Photobacterium damselae subsp. damselae from diseased sea bass reared in the Black Sea. We uncovered a great genetic diversity in the Black Sea isolates of this pathogen, suggesting a multiclonal origin. We also demonstrate for the first time that these isolates bear pathogenic potential for sea bass cultures by virulence challenges.
INTRODUCTION

*Photobacterium damselae* subsp. *damselae* is a marine bacterium of the family *Vibrionaceae* that is recognized as a pathogen for a wide variety of aquatic animals including fish, molluscs and crustaceans. In addition, it is a pathogen of concern for humans, being capable of causing fatal infections (1). Most of the reported infections in humans are originated after wounds inflicted during handling of fish and fishing tools or by exposure to marine animals or seawater. Notably, it is a primary pathogen of fish species of economical importance in aquaculture. During the last decades it has been isolated as causative agent of outbreaks in turbot (*Scophthalmus maximus*) (2), gilthead sea bream (*Sparus aurata*) (3) and rainbow trout (*Oncorhynchus mykiss*) (4), among others. Recently, this pathogen started to be isolated from outbreaks of newly cultured species of marine fish, observations that led to its consideration as an emerging pathogen in aquaculture (5). Outbreaks in sparid fish were reported recently in the Mediterranean area (6-9), affecting gilthead sea bream, white sea bream (*Diplodus sargus*) and redbanded sea bream (*Pagrus auriga*).

In addition, recent reports mentioned the isolation of *P. damselae* subsp. *damselae* from outbreaks in sea bass (*Dicentrarchus labrax*) cultures in Spain (7), Egypt (9), and Tunisia (10). Recently, this pathogen was isolated from moribund sea bass cultured in the Turkish Black Sea along a three-month period, coincident with episodes of fish mortality and with water temperatures close to or above 24°C (11). Thus, surveillance of *P. damselae* subsp. *damselae* in marine fish cultures has shown a notable increase in its geographical distribution during the last years, causing disease outbreaks in countries where it had never been reported before. However, despite this increasing number of reports on isolation of this pathogen from sea bass, and from novel geographical locations, the studies at the genetic level to identify the presence of virulence markers have been scant. In addition, little is known about the epidemiology of the isolates and about the clonality of the populations causing the recent outbreaks.

Recently, the virulence gene content of this subspecies started to be elucidated. Currently it is recognized that highly hemolytic isolates harbour a 153-kb virulence plasmid named pPHDD1, encoding the two hemolysins damselysin (Dly) and HlyA, the latter being recently renamed phobalysin (PhlyP) due to its differential characteristics (13). In addition, the hemolytic isolates encode in chromosome I a hemolysin dubbed HlyA, being responsible for the hemolytic activity of pPHDD1-negative strains (14). It is acknowledged that the synergistic effect of Dly with either...
PhlyP or HlyA<sub>ch</sub> is responsible for maximum virulence in a turbot model (14). Interestingly, only a fraction of<sup>1</sup> <i>P. damselae</i> subsp. <i>damselae</i> strains harbour pPHDD1 (15), and plasmidless strains have been routinely isolated from recent outbreaks in sparid fish (8). To date, no information is available on the virulence gene baggage of <i>P. damselae</i> subsp. <i>damselae</i> isolates from sea bass, and there is no information on the prevalence of pPHDD1 plasmid further than in some human and turbot isolates (15). The aim of the present work was to characterize a collection of <i>P. damselae</i> subsp. <i>damselae</i> strains isolated from sea bass during a survey in two rearing facilities in the Black Sea in Turkey, to carry out the study of their virulence gene baggage and to assess the pathogenicity potential for sea bass. The interest of this study is increased by the fact that the Black Sea is a particular environment, narrowly connected to other seawater masses, with warm temperatures and salinity levels below 18‰. This study constitutes the first report on molecular characterization of this pathogen isolated from disease outbreaks in the Black Sea, and our results indicate a multiclonal origin of the <i>P. damselae</i> subsp. <i>damselae</i> isolates in this area.

**MATERIALS AND METHODS**

**Bacterial strains isolation and characterization, and culture conditions.** In a previous study aimed at determining the occurrence and frequency of bacterial pathogens in cultured sea bass, spleen and kidney of fish belonging to 2009, 2010 and 2011 age classes were sampled for bacteriological analyses in 2011 (11). Three cages from each of two farms (Farm A, Persembe, in the Ordu Province and Farm B, Yomra, in the Trabzon Province, in the Turkish coast of the Black Sea) (Fig. 1) and 10 to 24 sea bass per cage were sampled. Water temperature ranged from 17.4°C to 25.8°C from June to October, although most samples were collected at water temperatures above 24.5°C. Salinity ranged from 16 to 17‰. A total of 14 strains (Table 1) were presumptively assigned to <i>Photobacterium damselae</i> subsp. <i>damselae</i>. <i>P. damselae</i> subsp. <i>damselae</i> and subsp. <i>piscicida</i> isolates used in this study (Table 1 and Table 2) were routinely grown at 25°C on tryptic soy agar supplemented with 1% NaCl (TSA-1). For hemolysis assays on agar plates, a single colony of each strain grown on a TSA-1 plate was picked with the tip of a rounded wooden pick and seeded on Sheep blood agar plates (Oxoid), and pictures were taken after 24 h. Motility assays were carried out using motility agar, which consisted of LB broth supplemented with 0.25%
bacteriological agar. For this assay, a single isolated colony of a 18h-culture agar plate for each strain was picked with a sterile plastic tip and stabbed into the motility agar. Pictures were taken after 24h. This procedure was repeated three times to ensure that the motility radius of the strains were reproducible.

**DNA techniques.** Genomic DNA was extracted with the Easy-DNA kit (Invitrogen). Relevant PCR primers used in this study are listed in Table 3. The genetic context of hlyA<sub>ch</sub> gene was amplified by inverse PCR starting from conserved flanking genes. Nearly complete 16S gene was amplified using primers PA forward (corresponding to *E. coli* 16S rRNA gene positions 8-27), and PH reverse (*E. coli* 16S rRNA gene positions 1522-1541) as previously described (16). PCR reactions were routinely performed with Kapa Taq DNA polymerase (Kapa) using a T-gradient termocycler (Biometra) with the following thermal cycling conditions: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 57 °C for 30 s and an elongation step of 1 min at 72 °C per kb. DNA sequences were determined by Sanger sequencing and a capillary DNA Sequencer ABI 3730xl (Applied Biosystems).

**Experimental infection.** To test the pathogenicity of Black Sea isolates of *P. damselae* subsp. *damselae* obtained from sea bass, two strains (164dp and 64bp) were selected for experimental infection of sea bass, representing the two categories of hemolytic and non-hemolytic isolates, respectively. In addition, the highly-hemolytic and pPHDD1-harbouring strain RM-71 (2, 12) was used in the challenges. Fish were obtained from IGAF (Illa de Arousa, Galicia, Spain). Groups of 10 fish (6 ± 1.2 g) per strain tested and per dose were acclimated in 100-l aquaria at 24°C for one week before the challenges were performed. The virulence tests were conducted by intraperitoneal injection of bacterial suspensions. Fish were challenged with 0.1 ml of bacterial suspensions of each strain in 0.85% NaCl solution at two different doses of 5.4 × 10<sup>5</sup> and 3.4 × 10<sup>7</sup> CFU/fish. A control group was inoculated with the same volume of sterile 0.85% NaCl solution. Fish mortality was recorded daily for 10 days post-challenge. Re-isolation on TSA and TCBS agar plates and identification of the bacteria from the kidney of dead fish were performed. Colonies were confirmed by the subsp. *damselae*-specific ureC gene PCR test as previously described (17). All the protocols of animal experimentation used in this study have been reviewed and approved by the Animal Ethic Committee of the Universidade de Santiago de Compostela.
Molecular Phylogenetic analysis. The evolutionary history of the strains based on the toxR gene was inferred using the Neighbor-Joining method (18). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (19) and are in the units of the number of base substitutions per site. The analysis involved 28 toxR nucleotide sequences. Evolutionary analyses were conducted in MEGA6 (20).

Accession numbers. DNA sequences have been deposited in GenBank database under accession numbers KU760730 to KU760757 and KU896559 to KU896561.

RESULTS

Taxonomic verification and phenotypic diversity of P. damselae subsp. damselae sea bass isolates from the Black Sea. In a previous study (11) two sea bass farms distantly located from each other in the Turkish coast of the Black Sea (Fig. 1) undergoing fish mortalities, were sampled for presence of bacteria in kidney and spleen. 14 presumptive isolates of P. damselae subsp. damselae were isolated from erratically swimming fish, and half of them showed nodules in both kidney and spleen. The collection included isolates from three different fish ages, namely fish born in 2009, 2010 and 2011, respectively (Table 1). In the present study, these 14 isolates were subjected to genotypic and phenotypic characterization. Complete 16S rRNA gene sequences presumptively identified the 14 isolates as P. damselae subsp. damselae, showing 100% identity to the consensus 16S gene sequence of the type strain ATCC33539 (GenBank Acc. No. X74700) (data not shown). Although the 16S rRNA sequence is identical between P. damselae subsp. damselae and P. damselae subsp. piscicida subspecies (16), characteristics as the growth at 37°C and the ability to grow on TCBS agar clearly distinguished these isolates as belonging to subsp. damselae. The positive amplification of subspecies-specific ureC gene encoding a subunit of urease enzyme (17) in all the strains with the exception of isolate 70dps, provided additional evidence of the identification of the isolates as subsp. damselae (Table 1).
Differences were evidenced among the 14 isolates in sucrose fermentation in TCBS. Although strains of this pathogen are typically considered to produce green colonies on TCBS medium, we found that 6 sea bass isolates exhibited yellow colonies (Table 1). In addition, we observed diversity in the motility phenotypes of the isolates when assayed in motility agar: one strain was non-motile (70dps) and two other strains exhibited little motility (144bp and 111bp), with the remaining strains showing different levels of spreading in motility agar after 24 h (Fig. 2a). All these observations initially suggested that the P. damselae subsp. damselae isolates from the Black Sea might derive from different genetic lineages coexisting in this geographical area, rather than constituting a clonal lineage derived from a single virulent clone.

Hemolytic activity and hemolysin gene baggage. The three main recognized virulence factors of P. damselae subsp. damselae so far are hemolysin genes. The two hemolysins Damselysin (Dly) and the pore-forming toxin PhlyP are both encoded in the pPHDD1 plasmid, whereas a third hemolysin HlyAch is encoded in chromosome I. In a previous study we found that P. damselae subsp. damselae strains can be divided into categories depending on the width of their haloes of β-hemolysis (LH, large halo; MH, medium halo; SH, small halo; NH, non-hemolytic) (12). In the present study we found that the sea bass strains exhibited either a small β-hemolytic halo (SH) (11 isolates) or absence of β-hemolytic halo (NH) (3 isolates) in sheep blood agar plates, in contrast to the pPHDD1-harbouring highly-hemolytic strain RM71 isolated from turbot that we used as a control of LH phenotype (Fig. 2b). Thus, the hemolytic tests seem to indicate that none of the sea bass isolates from the Black Sea contain the pPHDD1 plasmid, and suggest that the 11 SH isolates might harbour the hlyAch gene in their genomes. In order to test these hypotheses, we carried out a PCR screening of the dly, phlyP and hlyAch genes and the results were compared with their hemolytic phenotypes. We found that all the 14 Black Sea strains tested negative for dly and phlyP genes (Table 1) and they all yielded negative results for a PCR test targeted to the pPHDD1 replication origin (data not shown). These results thus indicate that pPHDD1 plasmid is not present in the Black Sea isolates from sea bass.

We therefore tested for presence of hlyAch gene, using different primer combinations that allow for detection of partial and complete gene, respectively. Interestingly, we found that the 11 SH strains tested positive for both partial and complete hlyAch gene, whereas the three NH strains 64bp, 89dp and 144bp tested negative for the complete
gene but still exhibited partial amplification products of this gene. These results thus suggested that the three non-hemolytic strains might contain hlyAch pseudogenes.

The non-hemolytic *P. damselae* subsp. *damselae* strains have undergone genetic modifications. In a previous study (15) we reported that the genomic context of hlyAch contained an unexpected genetic diversity, and suggested that hlyAch is located in an unstable chromosomal region, which in some cases contain typical features of mobile DNA. Invariably, the chromosomal region upstream hlyAch promoter includes two serine tRNA genes and the conserved gene kefA encoding a putative ion channel (VDA_002421 in ATCC33539 genome annotation), present in all *P. damselae* subsp. *damselae* strains analyzed to date. Therefore, to investigate the genetic basis of the absence of a β-hemolytic phenotype in strains 144bp, 64bp and 89dp, we accomplished the complete sequencing of the genome region comprised between the hlyAch transcriptional terminator and the 3’-end of the conserved gene kefA in all the isolates.

We found that the 11 hemolytic SH strains, plus the two NH strains 64bp and 89dp invariably contained the structure of hlyAch-tRNA-tRNA-kefA genes almost identical to the sequence previously described in the strain RM71 (Fig. 3). Strain 144bp, on the contrary, showed a different genetic context with two additional ORFs between hlyAch and kefA, and was 99% identical to the homologous region previously reported in *P. damselae* subsp. *damselae* strain LD07 (15), a Spanish isolate from gilthead sea bream from 1991 (3).

Regarding the three NH strains 64bp, 89dp and 144bp we found that their lack of hemolysis was attributable to the presence of an insertion sequence element of the IS10 family disrupting the coding sequence of hlyAch gene (Fig. 3). This IS10 showed to be identical to an IS10 element previously described in a multicopy-fashion in the conjugative plasmid pAQU1, from a *P. damselae* subsp. *damselae* isolate from Japan (21), and different from the previously reported IS element that disrupts hlyAch gene in *P. damselae* subsp. *damselae* isolate 501H (15).

Interestingly, we uncovered that the insertion of IS10 took place at exactly the same position in the two isolates 64bp and 89dp, corresponding to position 1586 in the open reading frame of hlyAch gene, in a codon that encodes for Asn529 in the HlyAch protein, whereas the insertion of IS10 in strain 144bp took place in the codon that encodes for Tyr410 in the HlyAch protein (Fig. 3). Altogether, the data on the hlyAch gene context
point at the existence of different genetic lineages among the *P. damselae* subsp. *damselae* isolates from sea bass reared in the Black Sea.

**toxR** gene-based phylogenetic analysis: evidences for a multiclonal origin of the Black Sea population of *P. damselae* subsp. *damselae* associated to sea bass farms.

We wanted to obtain a further evidence of the multiclonal origin of these isolates, since this study represents the first demonstrated report on the occurrence of this pathogen in the Black Sea. The **toxR** gene, encoding a transmembrane transcriptional regulator involved in many aspects of virulence regulation, is considered a valuable molecular clock for fine-tuned discrimination of taxa within the *Vibrionaceae* due to its high variability, and a 9% divergence had been previously reported between subsp. *damselae* and subsp. *piscicida* **toxR** gene sequences (22). Therefore, we amplified and sequenced the complete **toxR** gene in all the isolates from the Black Sea, and for comparative purposes we also sequenced this gene in other subsp. *damselae* and subsp. *piscicida* isolates from our laboratory collection. The comparative phylogenetic analysis revealed a high level of identity among the subsp. *piscicida* isolates, clustering together in a well-defined branch (Fig. 4). This observation is of special interest, since these subsp. *piscicida* strains comprise isolates from Europe as well as from Japan, and some of them were isolated from distant locations and with many years of difference in between (Table 2). Interestingly, we observed that this gene constitutes a pseudogene in all the seven subsp. *piscicida* isolates, due to the existence of a stop codon that truncates the open reading frame (data not shown).

In addition, we observed that six *P. damselae* subsp. *damselae* isolates from closely-related epizootic outbreaks reported between 1987 and 1991 in turbot farms in Galicia (NW Spain), were closely associated. Four of them (RG-91, RG-153, RG-214 and RG-191) clustered together with **toxR** sequences 100% identical, although they were isolated in different years between 1987 and 1999. On the contrary, the subsp. *damselae* isolates from the Black Sea showed a different picture, with a dispersed distribution in several branches of the phylogenetic tree constituting six different groups. One group (cluster A) included six isolates with sequence similarities between their **toxR** sequences of 100%. Notably, among the 8 isolates that were not included in cluster A, the pair 70dps/111bp (Cluster B) and the pair 64bp/89dp (Cluster C) forming two well-defined branches respectively, showed a conspicuous phenotypical trait each. Cluster B is represented by the two strains with less motile phenotypes, whereas Cluster C includes
the two non-hemolytic isolates that share the same IS10 insertion mutation in the hlyAch gene. Interestingly, strain 125dy showed to be closely associated with the turbot isolates from the NW Spain outbreaks between 1987-1991 whereas 162bp and 189bp (Cluster D) were phylogenetically associated with strain Lb07070501R isolated from sea bass in the Mediterranean area, and to the subsp. piscicida isolates. Strain 144bp, as mentioned above, has a genetic context upstream hlyAch gene almost identical to the previously described in strain LD-07, and the toxR gene sequences of these two strains were also 100% identical (Fig. 4).

Another result that deserves attention is the distribution of sequence types throughout the year classes of sea bass in the farms. 2009 year class fish were at the fish farm for three years and bacteria were isolated in 2011, whereas 2011 year class fish were juveniles and they likely become infected after they were transferred. Cluster A includes isolates from year classes 2010 and 2011 (Table 1), suggesting that fish from different years might have been reinfected by bacteria already present in the fish farm environment. As can be deduced from the phylogenetic tree, the 2011 year class fish in the Persembe farm were infected by as many as 3 sequence types (isolates in clusters A, C and D). Regarding the geographical location, it is also pertinent to say that the two isolates from the Yomra farm (82dy and 125dy) do not represent a single clone but they branch far apart from each other.

Virulence for sea bass of P. damsela subsp. damsella Black Sea isolates. In order to assess the virulence of P. damsela subsp. damsella for sea bass, one strain with small hemolytic halo (164dp) and one strain without hemolytic halo (64bp) were used in challenge studies. We also included in the challenges the highly hemolytic strain RM-71 (2) were the pPHDD1 virulence plasmid was first described (12). All the challenges were conducted in fish kept at a water temperature of 24°C and using two different doses of 5.4 × 10^5 and 3.4 × 10^7 CFU/fish. Strain RM-71 containing the pPHDD1 virulence plasmid was highly virulent for sea bass, with mortality rates of 100% at the two doses (Fig. 5). Remarkably, this strain caused the death of 100% of the animals in less than 24 h, indicating not only a strong pathogenic potential but also a lack of stringent host-specificity, since RM-71 was isolated from a turbot epizootics in 1988 (2). The virulence data obtained with strain 164dp (small hemolytic halo and hlyAch positive) demonstrated the pathogenic potential for sea bass of the plasmidless strains isolated from the Black Sea (Fig. 5). The mortality rates for 164dp were 20% and 80%,
at the doses of $5.4 \times 10^5$ and $3.4 \times 10^7$ CFU/fish, respectively, and the temporal progression of the mortality differed with respect to that of RM-71 strain. For the non-hemolytic strain 64bp (hlyAch gene disrupted by an insertion element) the mortality rates at the same two assayed doses were 0% and 20%, indicating that this strain only exhibits a low virulence degree under the conditions tested. Almost all dead fish exhibited hemorrhagic areas around the mouth, fins and anus. *P. damselae* subsp. *damselae* could be re-isolated from the kidney of all dead fish post-challenge, and colonies were proven to belong to this subspecies as they tested positive in a colony-PCR for the subspecies-specific *ureC* gene (data not shown). These results demonstrate the pathogenic potential of the plasmidless *P. damselae* subsp. *damselae* from the Black Sea for sea bass.

**DISCUSSION**

Turkey is one of the major sea bass producers in Europe, and this fish is the second most favourable cultured fish species, after rainbow trout, in the Turkish coastal brackish waters of the Black Sea. Several infectious diseases affect the cultivation of sea bass in this environment, including viruses as viral erythrocytic infection virus VEIV (23), parasites as *Diplectanum aequans* (24), and bacteria as *P. damselae* subsp. *damselae* and *Aeromonas veronii* biovar sobria (11). *P. damselae* subsp. *damselae* is being increasingly isolated from disease outbreaks in sea bass, as well as in gilthead sea bream and other sparid species in the Mediterranean area. These reports led to the consideration of *P. damselae* subsp. *damselae* as an emerging pathogen in marine aquaculture (5). The first report of presence of this pathogen in the Black Sea (23) was not accompanied by a genetic characterization of the isolates. More recent reports described the presence of this bacterium in sea bass in the Black Sea (11). Until now, no studies had been conducted to characterize sea bass isolates of this emerging pathogen, and indeed there was a complete lack of data on virulence challenges of *P. damselae* subsp. *damselae* for sea bass, since most data on virulence were obtained using turbot (2), redbanded sea bream (8) and rainbow trout (4) as models of infection. We determined that the collection of Black Sea isolates of this pathogen exhibited variable phenotypes and genotypes. Although *P. damselae* subsp. *damselae* is recognized as a urease positive subspecies in most reports (2-4, 25, 26), previous studies already reported the existence of urease negative isolates (27), and in our study we
found that isolate 70dps was the unique that tested negative for *ureC* gene. Moreover, albeit strains of this pathogen are typically reported to exhibit green colonies on TCBS medium (2, 9, 25, 28), we found that 6 Black Sea sea bass isolates produced yellow colonies on TCBS, an observation to be added to a previous study that also reported the existence of yellow colonies of this pathogen (29). Interestingly, we observed diversity in the motility of the Black Sea strains as demonstrated by motility agar assays, constituting the first reported evidence of variability in this phenotypical trait in this pathogen to date. Since a large number of genes are supposed to be involved in flagellar motility in *Vibrionaceae*, including structural and regulatory flagellar genes as well as genes of chemotaxis systems (30), this observation also points at the existence of genetic heterogeneity among the Black Sea populations of *P. damselae* subsp. *damselae*.

Virulence experiments using the highly hemolytic strain RM-71 indicate that pPHDD1 plasmid is a major virulence factor for sea bass, as this strain caused 100% fish death in the first 24h after inoculation. The two pPHDD1-encoded hemolysins Dly and HlyApl (recently renamed as PhlyP) are considered major virulence factors for turbot, and HlyA<sub>ch</sub> is believed to contribute to virulence for turbot to a lesser extent (14). Here we have demonstrated that pPHDD1 plasmid is absent in all the Black Sea isolates from sea bass, confirming a trend anticipated in a recent study where pPHDD1 plasmid was restricted to a 20% of 44 isolates tested (15). It is becoming evident that pPHDD1-negative isolates of *P. damselae* subsp. *damselae* are pathogenic for numerous fish species. Recent studies reported that *P. damselae* subsp. *damselae* strains lacking pPHDD1 were virulent in a redbanded seabream model (8). Another study carried out with *P. damselae* subsp. *damselae* strains isolated from rainbow trout in Denmark revealed over 1000 × difference in LD<sub>50</sub> between highly hemolytic strains (presumptively harbouring pPHDD1) and low hemolytic strains (presumptively encoding only HlyA<sub>ch</sub>), but still these low hemolytic strains were virulent for trout (4).

In this regard, we have here demonstrated that the pPHDD1-negative isolates from the Black Sea environment bear pathogenic potential for sea bass, and virulence was greatly diminished in the strain bearing a mutated hlyA<sub>ch</sub> gene version. Taken together, the evidences clearly support the hypothesis that pPHDD1-negative *P. damselae* subsp. *damselae* isolates pose a health risk for many fish species of interest in aquaculture and point at a role of HlyA<sub>ch</sub> hemolysin in virulence. Our discovery that the 3 non-hemolytic isolates from the Black Sea contain pseudogenes of hlyA<sub>ch</sub> gene reinforces the
hypothesis that the chromosomal region encoding HlyA,ch is a hotspot for recombination
and IS element insertion in the *P. damselae* subsp. *damselae* genome, and opens the
question of why a gene encoding a virulence factor of this subspecies is so prone to
undergo genetic inactivation events. To date only two *P. damselae* subsp. *damselae*
isolates had proved negative for this gene, and were isolated from distantly located
places (15).

We used the gene encoding the transmembrane transcriptional regulator ToxR as a
phylogenetic marker in order to provide clues on the phylogeography of the *P.
damselae* subsp. *damselae* Back Sea isolates. The *toxR* sequence is being increasingly
used as a valuable molecular clock for fine-tuned discrimination of taxa within the
*Vibrio*aceae due to its high variability and the existence of intra-subspecies variation
in *P. damselae* subsp. *damselae* (22, 31). The phylogeny based on *toxR* gene unveiled
the existence of genetic heterogeneity among the Black Sea isolates, and we found
interesting correlations between identity at the *toxR* gene level, and occurrence of the
same phenotypic or genotypic features. Of special interest is the observation that strain
144bp shared the same genetic context upstream the *hlyA,ch* locus with strain LD-07, as
well a 100% identity in the *toxR* gene. These two strains were isolated thousands of
kilometers apart, with 20-year difference and from different host species. Similarly, the
two non-hemolytic strains 64bp and 89dp contain exactly the same *IS10* insertion
disrupting *hlyA,ch* gene, and their *toxR* gene sequences are 99.9% identical. The
observation that Cluster A isolates share 100% identical *toxR* gene sequences suggests
that this specific group of strains might be clonal in origin. This being the case, the
differential features observed between strain 144bp and the rest of Cluster A strains in
the *hlyA,ch* locus might correspond to recent genetic events. On the one side, the
acquisition of a *IS10* element disrupting *hlyA,ch* is a distinctive and unique feature among
the 14 Black Sea isolates, and might constitute a recent transposition event. On the other
side, the presence of two open reading frames upstream *hlyA,ch* in 144bp but not in the
rest of Cluster A isolates might be explained by a gene loss event, since that DNA
sequence is flanked by two large sequence repeats that are believed to constitute
hotspots for gene recombination (15).

The multiclonality hypothesis is reinforced by the observation that the *toxR* gene
sequences among the different Clusters of *P. damselae* subsp. *damselae* isolates from
the Black Sea show a much higher degree of variability among them than the Cluster of
subsp. *piscicida* strains or the Cluster of turbot isolates from Spain of subsp. *damselae*. 
The phylogenetic data, together with the phenotypic diversity and the existence of different gene patterns in the vicinity of the hlyA ch loci, clearly indicate that the P. damselae subsp. damselae circulating in the environment of sea bass rearing facilities in the Turkish Black Sea did not originate from a clonal spread of a single strain but from the simultaneous occurrence of several clones. Previous studies using different molecular typing methods pointed at the idea that P. damselae subsp. damselae is a genetically diverse subspecies. A study analyzing 50 isolates from seafood in Taiwan encountered as many as 42 typeable profiles by PFGE (32). Similarly, a high number of PFGE profiles were reported among 16 isolates from rainbow trout in Denmark, a finding that led the authors to conclude that the disease outbreaks in Danish rainbow trout were caused by a multiclonal population of this pathogen (4).

The genetic diversity that we found among bacteria from the two sea bass farms in the Black Sea suggests that the infectious agent was already present in the environment, likely waiting for the appearance of stressful conditions, as for example a rise in the water temperature, in order to cause infection. Global warming is causing that bacterial species previously unreported in certain ecological areas, are now being increasingly reported, and not only as mere environmental bacteria, but as pathogens of either humans or animals, or both (33). Actually, the pathogen was only recovered from sea bass kidney and spleen in the period between July-October, i.e, in the warm season, with water temperatures ranging from 22.9 to 25.8°C in most sampling points. The presence of stressful conditions and of changes in the environmental variables, namely temperature (2, 25), is increasingly suggested to be associated with the pathogenicity of P. damselae subsp. damselae in fish. It is thus possible that P. damselae subsp. damselae behaves as an opportunistic, not clonal pathogen for sea bass in the Black Sea environment.

ACKNOWLEDGEMENTS

This study was supported by grant AGL2013-48353-R, from the Ministry of Economy and Competitiveness (MINECO) of Spain (cofunded by the FEDER Programme from the European Union). Mateus S. Terceti thanks the Brazilian Ministry of Education and CAPES (Coordenaçao de Aperfeiçoamento de Pessoal de Nível Superior) for a predoctoral studentship. We thank Bernardo Fernández Souto and the Instituto Galego de Formación en Acuicultura (IGaFA) (Illa de Arousa, Galicia, Spain) for their valuable support in providing sea bass for the virulence challenges.
REFERENCES


**FIGURE LEGENDS**

**FIG 1.** Geographical location of Farm A (Persembe) and Farm B (Yomra) in the provinces of Ordu and Trabzon, respectively, in the Turkish coast of the Black Sea. (Map created with QGIS version 2.14.1 Essen.)

**FIG 2.** (A) Swimming motility phenotypes of the 14 *P. damselae* subsp. *damselae* strains isolated from sea bass in the Turkish coast of the Black Sea, after 24h growth in
motility agar. The motility radius were reproducible in three independent repeats of the experiment, although only one experiment is shown. (B) Hemolytic phenotypes of the 14 strains on sheep blood agar. For comparative purposes, the highly hemolytic pPHDD1-harbouring strain RM-71 is included. Note that, in comparison to RM-71 strain, all the Black Sea strains are either weakly hemolytic (hemolysis due to hlyA<sub>ch</sub> gene) or non-hemolytic (strains 144bp, 64bp and 89bp contain hlyA<sub>ch</sub> pseudogenes). Scale bar: 1 cm (motility assays were conducted in 15cm Petri dishes and hemolysis assays in standard 9cm dishes).

**FIG 3.** Variable chromosomal region containing the hlyA<sub>ch</sub> gene in the 14 <i>P. damselae</i> subsp. <i>damselae</i> isolates from sea bass in the Black Sea analyzed in this study. Conserved genes encoding two serine tRNAs and KefA protein are present in all the strains. The hemolytic phenotypes are depicted in the right panels. The 11 isolates with small hemolytic halo (SH) contain a complete hlyA<sub>ch</sub> gene (A). In the two non-hemolytic strains 64bp and 89dp, hlyA<sub>ch</sub> is disrupted by an insertion sequence (IS10) element (B). The non-hemolytic strain 144bp, in addition to an IS10 element disrupting hlyA<sub>ch</sub>, contains two open reading frames (CDS_11 and CDS_12) between hlyA<sub>ch</sub> and the tRNAs (C). Ψ<sub>hlyA<sub>ch</sub></sub> denotes hlyA<sub>ch</sub> pseudogene.

**FIG 4.** Neighbour-joining tree showing the phylogenetic relationships of the 14 Black Sea sea bass isolates of <i>P. damselae</i> subsp. <i>damselae</i> (names in red) to other <i>P. damselae</i> subsp. <i>damselae</i> (names in black) and subsp. <i>piscicida</i> (names in blue) strains. Numbers at the nodes show bootstrap values (%). Only bootstrap values >70% are shown.

**FIG 5.** Survival (%) of sea bass intraperitoneally challenged with two different doses of three <i>P. damselae</i> subsp. <i>damselae</i> isolates (n=10 fish per dose and strain). RM-71 is a highly virulent, pPHDD1-harbouring turbot isolate from Galicia (NW Spain). 164dp (hemolytic, hlyA<sub>ch</sub>-positive) and 64bp (non-hemolytic, hlyA<sub>ch</sub>-negative) are two sea bass isolates from the Turkish Black Sea characterized in the present study. Control group of 10 fish inoculated with saline solution achieved a 100% survival (data not shown).
A

B

C

11 SH isolates

64bp
89dp

144 bp